bute biologically-active hormonal substances within the brain, since many researchers have found various hormones in the CSF, such as LHRH and thyrotropin-releasing hormone (TRH) [56], oxytocin and vasopressin [57], and melatonin [58]. The concentrations of these hormones varied according to various physiological statuses. Further, single intraventricular injections of LHRH and TRH increased the amplitudes and frequency of electroencephalographic (EEG) activity recorded from the brain of a hibernating Japanese toad (Fig. 4) [59]. The effective dose of 1 μg needed for enhancement of EEG activity through a single intraventricular injection of LHRH or TRH was much less than that needed for systemic injections. It is thus possible that the CSF-contacting neurons whose dendritic processes protrude into the preoptic recess detect changes in ventricular hormonal status and motivate the neuronal circuitry in preparation for mating behavior in pre-breeding anurans.

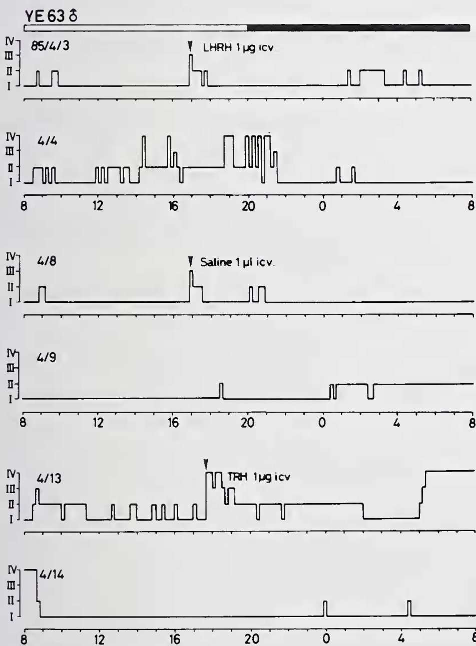

Fig. 4. Effects of intraventricularly injected LHRH and TRH on EEG activity of a hibernating Japanese toad. According to amplitudes and frequency, EEG activity was categorized into 4 levels: I, resting; II, awake; III, active; and IV, very active. Each level corresponds well to a behavioral state. Note that both LHRH and TRH induced dual fast and slow enhancements of EEG activity. Although the EEG record is not shown, the pattern of LHRH-induced fast response includes highly synchronized bursting waves that were not observed upon injection of control saline. (Fujita, thesis, Saitama University)

Blood Capillary (BC)-Contacting Neurons

The presence of BC-contacting neurons is incompatible with the general concept of the relations between brain neurons and capillaries. Blood capillaries in the vertebrate brain are generally surrounded by astrocytic endfeet with an intervening basement membrane, so that brain neurons, even fish hypothalamic neurosecretory cells, are separated from the vascular endothelium [60]. Nonetheless, neurosecretory cells which directly come into contact with blood capillaries were shown in the toad preoptic nucleus [61]. Recently, it was found that a considerable number of peptidergic neurons come into contact with blood capillaries with only an intervening basement membrane in the APON of both the bullfrog and the Japanese toad [36].

BC-contacting neurons send their dendrites laterad toward the preoptic white matter. Although arborization is rather poor, the dendrites usually bifurcate several times and form dendritic fields. There, many axon terminals form synapses on the dendritic spines of these neurons. It is highly probable that APON neurons receive the input signals of the afferent fibers mainly through the dendritic synapses in the preoptic white matter along the border of APON cell mass, since Halpern [62] noted that terminal degeneration by the telencephalic lesions was located along the lateral edges of cell masses in the frog hypothalamus. The single BC-contacting neurons thus detect changes in titers of blood-born hormones, preferably sex steroid hormones which have activational effects on APON neurons, and further receive neuronal input signals through dendritic synapses to integrate hormonal and neural signals concerned with the initiation of sex behavior.

AFFERENTS OF THE APON

Retrograde Horseradish Peroxidase (HRP) Study

It is important to know what neuroanatomic afferent relations the APON has with other parts of the brain. Such information is requisite for better understanding of the sensory modalities and activating or inhibiting pathways that might trigger or modulate sexual behavior through the PON. Thus, the afferents of the APON were examined in *Rana pipiens* (Urano and Gorbman, unpublished) and *Bufo japonicus* [63] using the retrograde HRP method, which is a particularly useful tool in studies of neural connections.

Evidence of retrogradely transported enzymatic activity was observed in perikarya and neuropil in the following brain regions: the ventro-medial limbic cortex, the posterior part of the preoptic nucleus including the magnocellular part, the infundibular nuclei, the thalamic area, the subtectal and tegmental regions including the reticular formation, and the rhombencephalic central gray. Neurons in these regions appear to send their axons to the APON mainly *via* the medial and lateral forebrain bundles. Localization of some HRP-labeled perikarya and fibers coincides with that of immunoreactive perikarya and fibers containing either LHRH, vasotocin or TRH which have been considered to project to the APON [64, 65].

Particular HRP-labeled loci in the ventro-medial limbic cortex included the nucleus medialis septi, the nucleus lateralis septi, the nucleus accumbens septi, the amygdala pars medialis and the nucleus of the diagonal band of Broca. The amygdala-preoptic tract may exist in all vertebrate classes from cyclostomes to mammals [66]. The septal projection to the preoptic area in the leopard frog and the Japanese toad has an apparently homologous relationship to a similar pattern in the lizard [67] and the rat [68, 69]. Although in anurans, the physiological significance of amygdaloid and septal projections to the APON is not clear at present, it is possible that these projections are concerned with the control of sexual behavior as has been claimed in mammals [70, 71].

HRP-labeled structures in the subtectal and tegmental regions were the nucleus anterodorsalis tegmenti mesencephali, the torus semicircularis, the nucleus posteroventralis tegmenti mesencephali, the nucleus isthmi and the mesencephalic reticular nuclei. Mesencephalic projections to the anterior hypothalamus are well known in amphibian brains [17, 19, 66, 72] as well as in other vertebrate classes [66, 73, 74]. The mammalian preoptic area is directly continuous with a vast nonspecific neuronal apparatus of the brain stem reticular formation [75]. In frog brains, the mesencephalic reticular system receives afferents from various parts of the brain, such as the telencephalon [62, 76], the optic tectum [77], and the superior olivary nucleus [78]. The presence of multimodal inputs suggests a nonspecific or generalized character of function of the anuran reticular formation as a possible activating or inhibitory regulatory system which may influence the neural substrate for mating behavior.

Chemical Neuroanatomy of the APON Afferents

Information on the chemical nature of APON afferents is important for the examination of control mechanisms of APON neuronal activity at the cellular and molecular levels.

The HRP study mentioned above showed the presence of HRP-labeled neurons in the magnocellular part of the PON, and in the nuclei infundibularis dorsalis and ventralis in the toad brain. These regions are rich in vasotocinergic and mesotocinergic neurosecretory neurons [22], and TRH neurons [65, 79], respectively. Jokura and Urano [64] verified that varicose ir-vasotocin fibers are found in the ventrolateral region of the APON where the APON neurons have their dendritic fields. Some ir-vasotocin fibers from the *vmc* protrude into the APON cell mass, and appeared to come into contact with somata of APON neurons. In Japanese toads, ir-TRH neurons were localized mainly in the nucleus infundibularis ventralis (NIV) [65]. Ir-TRH fibers arising from the NIV neurons project to the median eminence to form the hypothalamo-hypophysial tract. In addition, a considerable number of ir-TRH fibers innervate into the APON. In the APON, varicose ir-TRH fibers are scattered widely among the neuronal cell mass and the white matter.

Other important loci in the toad brain where HRP-labeled neurons were found include the nucleus medialis septi and the nucleus of the diagonal band of Broca. These loci contained many ir-LHRH neurons which project to the APON [64]. Most ir-LHRH fibers emanating from the nucleus medialis septi form a loose fiber bundle with those arising from the diagonal band of Broca. These ir-LHRH fibers, which have typical beaded features, project to the ventrolateral border of the preoptic gray.

In mammalian brains, peptidergic axonic processes form ordinary synapses [80] and en passant synapses with dendritic profiles [81]. Therefore, it is highly probable that varicose ir-vasotocin, ir-TRH and ir-LHRH fibers form ordinary or en passant synapses in the dendritic fields of APON neuron in the toad brain.

Functional Significance of the APON Afferents

The retrograde HRP study indicates that there are multimodal inputs to the APON from various regions of the brain. The septal nuclei, which send ir-LHRH fibers to the APON, receive olfactory inputs through the medial olfactory tract [82, 83], and the amygdala is innervated by projections from the accessory olfactory bulb [84]. These limbic nuclei, from which afferents to the APON arise, may relay olfactory signals to the APON neurons. In addition, the terminal nerve, which may function in odor processing, sends an ir-LHRH-ergic projection to the preoptic region in the tiger salamander and the bullfrog [85].

Visual cues can be conveyed through direct retinal projection to the suprachiasmatic part of the PON. This was clarified in the Japanese toad by use of a cobaltic lysine method (Shimotoso and Urano, unpublished). The presence of direct retino-preoptic projetion has also been supposed in the brain of *Rana temporaria* [25]. Acoustic signals which excite APON neurons may reach the preoptic region through at least two ascending pathways in the brain stem [78]. One is the pathway relayed through the nucleus oliva superior and the nucleus profundus mesencephali; the other is that relayed through the nucleus oliva superior and the torus semicircularis. The thalamo-preoptic connection is a possible pathway for transmission of tactile signals. Thus, the APON neurons may be influenced by various kinds of sensory inputs, although almost all sensory modalities are relayed and may be regulated either by sex steroid hormones or by neurohormones released from extrahypothalamic terminals of neurosecretory neurons [86, 87]. Since the electrical activity of many APON units was excited by iontophoretically applied LHRH, TRH and vasotocin (Fujita and Urano, in preparation), the APON neurons probably integrate various sensory inputs under the influence of peptidergic neurosecretory neurons, and then generate neural signals for the initiation of mate calling behavior.

SEASONAL VARIATIONS

Many anurans, especially those in the temperate zone, are typical seasonal breeders which spawn in spring or early summer. The neuroendocrine systems associated with reproductive behavior also show seasonal changes in their synthetic and secretory activities. In bullfrogs, the plasma level of luteinizing hormone (LH), which can increase androgen secretion from the testes [88], was elevated during the breeding season [89]. In the *Xenopus* hypothalamus, the contents of LHRH, which can stimulate pituitary gonadotropin release in bullfrogs [90] and plasma androgen levels in Japanese toads [91], varied seasonally in correspondence to reproductive physiological states [92]. Ishii and his collaborators measured circannual changes in plasma levels of various pituitary hormones [93, 94], thyroid hormones [95], adrenal steroids [96, 97] and sex steroids [98]. Most of these hormones showed marked increases in their plasma titers prior to or during the breeding season. Further, classical histochemical and morphometric studies showed seasonal morphological changes in the hypothalamo-neurohypophysial neurosecretory system in *Rana temporaria* [99, 100]. The results of these studies suggest the possibility that the APON neurons do show some seasonal changes in their morphological and functional features, since the activity of APON neurons was modulated by administrations of testosterone [14] and pituitary homogenates [53].

Seasonal Changes in the Volumes of PON Subnuclei

Seasonal variations in the volumes of several preoptic and amygdala subnuclei were found between hibernating and postbreeding Japanese toads [34]. The APON in the hibernating males was 125% larger than that in the post-breeding animals. The seasonal difference in the female APON was not statistically significant. In both sexes, the hibernating animals had larger ventral magnocellular parts of the PON, amygdala medialis and amygdala lateralis than the post-breeding animals did. The seasonal variation in the volumes of several subnuclei mentioned above may be due to hypertrophy of the neurons in these loci, since cell nulear volumes of PON neurons increase prior to the breeding season [100]. Morphological changes in the APON and the amygdala thus precede physiological and behavioral changes in the breeding season. A similar result was observed in the song control nucleus in the brain of the male canary which is larger during breeding season than in the fall when the animal is sexually inactive [101], probably because sex steroids induce dendritic growth in this nucleus [102].

Immunoreactivity of Neuroendocrine Cells

It is highly probable that neuronal input signals to APON neurons differ seasonally. Therefore, seasonal variations in LHRH, TRH and vasotocin that were localized in varicose afferent fibers to the APON were examined immunohistochemically in toad forebrains and neurohypophyses [65, 103]. The immunohistochemical technique utilized was the avidin-biotin-peroxidase complex (ABC) method, which is superior to the peroxidase-antiperoxidase method in a quantitative study. LHRH immunoreactivity (ir) was strong in both perikarya and fibers in animals captured in spring and autumn, while in summer animals, LHRH-ir was weak. Seasonal changes in TRH-ir were similar to those in LHRH-ir, while significant seasonal variations were not found in vasotocin-ir. The circannual changes in LHRH-ir appear to correspond with seasonal variations in plasma steroid levels reported by Inoue *et al.* [98]. This coincidence implies that LHRH and sex steroids can have synergistic effects on the control of APON neurons.

Effects of Castration

As is described above, testosterone may modulate neural activity of the APON to initiate reproductive behavior. Structures of the nervous system are modified by sex steroids during both fetal and adult periods in many vertebrate species [101, 102, 104]. In Japanese toads, the volumes of the APON and the amygdala in the male are larger than those in the female. Furthermore, the volumes of these nuclei and LHRH-ir also changed seasonally. These changes appear to correlate with the annual variation of plasma testosterone levels. Castration experiments, in which the role of testosterone in the control of the phenomena mentioned above was examined, showed that the effects of castration differ seasonally [105]. The volume of the amygdala medialis in autumn toads was significantly reduced by castration; however, the reduction in spring animals was not statistically significant. Meanwhile, castration did not modify LHRH-ir in the median eminence in either spring or autumn toads, although dense ir-LHRH fibers were observed in the mesencephalic tegmental region in the castrated spring toads but not in the autumn toads either intact or castrated. These results suggest that seasonal influences on the effects of castration were not uniform among the different brain loci.

COORDINATION OF NEURAL AND ENDOCRINE ACTIVITY

Temporal coordination of neural and endocrine events is a cruial requisite for successful reproduction. Plausible candidates for coordinating the brain and endocrine functions are LHRH-ergic and vasotocinergic neurosecretory systems, both of which send fine varicose fibers to various extrahypothalamic brain loci other than the median eminence and the pars nervosa [86, 87]. Both LHRH-ergic and vasotocinergic fibers innervate either sensory or motor centers concerned with reproductive behavior.

LHRH applied by microiontophoresis increased discharge rates of individual neurons in the septal

preoptic area of the rat [106], and in the APON of the Japanese toad (Fujita and Urano, in preparation). An intracellular study showed that LHRH can mimic slow excitatory postsynaptic potentials when applied to the sympathetic neurons in the bullfrog [107]. LHRH at the synaptic level may play a role in increasing neuronal excitability in the loci where LHRH fibers innervate. On the other hand, LHRH applied systemically or intraventricularly can stimulate the pituitary-gonadal axis to elevate plasma androgen levels in male Japanese toads (Fig. 5). This evidence suggests that LHRH simultaneously affects both the neuronal activity of the APON neurons as an excitatory neurotransmitter or neuromodulator and the endocrine events of the pituitary-gonadal axis as a hypothalamic releasing hormone.

The endocrine functions of vasotocin in amphibians are well documented in many endocrine textbooks. In addition, vasotocin and its homologues can excite unit-spike activity of neurons in the rat supraoptic and paraventricular nuclei [106, 108], the eel preoptic nucleus [109] and the toad APON (Fujita and Urano, in preparation). Vasotocin thus may facilitate the activity of many central neurons as a neuromodulator or a local hormone. The latter possibility is supported by the fact that 10^{-9}M vasopressin, comparable to the effective dose of vasopressin necessary for peripheral targets, can excite rat paraventricular neurons [108].

At present, it is difficult to account for the temporal discrepancy between LHRH-induced neural events (Fig. 4) and endocrine events (Fig. 5). When LHRH functions as a neurotransmitter or a neuromodulator, its influence on target

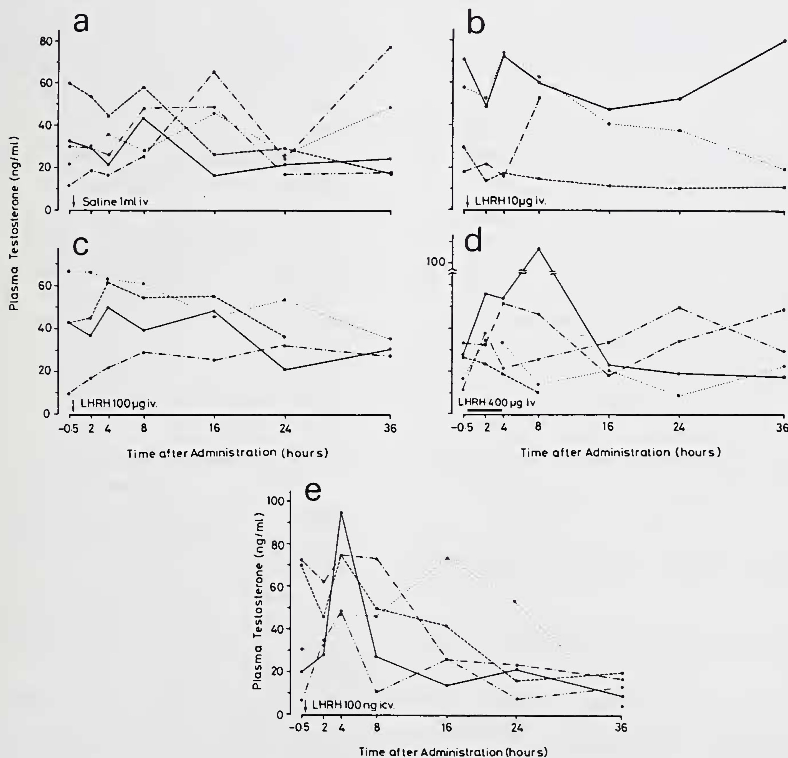

Fig. 5. Changes in the plasma testosterone levels after administrations of intravenous and intraventricular LHRH. Each curve represents a change in testosterone levels in an individual male toad. a, effects of intravenous saline as a control; b, single intravenous injection of 10 μg LHRH; c, single intravenous injection of 100 μg LHRH; d, continuous infusion of LHRH at a dose of 100 μg/hour for 4 hours; and e, intracerebroventricular administration of 100 ng LHRH. Note that the dose of intracranial LHRH which markedly elevated plasma testosterone was much less than that of intravenous LHRH. (Fujita, thesis, Saitama University)

neurons lasted for within the order of seconds or minutes. However, endocrine events, e.g., the secretion of androgen, take a much longer time. Since the APON neurons are sex steroid-sensitive and are excited by LHRH, some unknown intrinsic cellular mechanisms within the APON neurons and neurons having the same characteristics may regulate the above temporal discrepancy in order to complete seasonal breeding successfully.

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Activation of Respiration and Initiation of Motility in Rainbow Trout Spermatozoa

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ABSTRACT—It is well established that sperm motility of rainbow trout is initiated by the decrease in K^+ concentration surrounding sperm which triggers the intracellular cAMP-dependent initiation process. Present study showed that K^+ did not affect sperm respiration but inhibited flagellar movement and thus suggested that K^+ regulates sperm motility through its effect on flagellum. On the other hand, inhibitors of respiratory chain or uncoupler of oxidative phosphorylation affected sperm respiration and inhibited sperm motility, suggesting that energy producing system at mitochondria contributes to sperm motility. Motility was initiated even if O_2 was eliminated from dilution medium, although CO_2 suppressed both respiration and motility. This result suggested that sperm motility is not O_2-limited but CO_2 is responsible for the regulation of sperm motility through the activation of respiration. It is likely that regardless of K^+-dependent cAMP system at sperm flagella, there is another system at mitochondria: enhancement of respiration by the release from CO_2 suppression at spawning may relate to the initiation of sperm motility in rainbow trout.

INTRODUCTION

Spermatozoa are immotile in undiluted semen and initiate motility on dilution into appropriate medium. As factors to cause the phenomenon, many things in the seminal plasma have been proposed (see [1]). Rothschild [2] postulated that low O_2 tension in the seminal plasma is most likely responsible for the sperm immotility in the reproductive organ and that increase in O_2 tension surrounding spermatozoa at spawning causes initiation of motility. Carbon dioxide was also proposed as another possible factor from the results that CO_2 inhibits both respiration and motility in sea urchin sperm [3, 4]. Johnson *et al.* [4] also suggested that O_2 does not affect sperm motility, since motility initiation occurs when O_2 was eliminated by blowing N_2 gas over a thin layer of semen. These studies have focused on the contribution of energy supply system to the motility initiation; however it is still unclear which factor is the physiological initiator of sperm motility.

Morisawa and collegues recently proposed a motility initiation system from another point of view. They showed that motility of spermatozoa in salmonid fishes is suppressed by K^+ and spermatozoa become motile in the K^+ deficient medium [5]. However, K^+ can not inhibit motility of trout spermatozoa of which plasma membrane and mitochondria are removed with the detergent [6], implicating that the site of K^+ action is not mitochondria but flagella. By regulating flagellar motility with or without K^+, it is possible to separate the mitochondrial function from the flagellar function. Consequently, salmonid sperm seems to offer an especially convenient material for investigating which factor contributes to mitochondrial metabolism or flagellar mechanism in the initiation of sperm motility.

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For clarifying this point, we compared the respiration and motility in trout sperm in the presence or absence of K^+ and furthermore examined the effects of aerobic or anaerobic condition and CO_2 on the sperm respiration and motility. The results suggested that K^+ dependent initiation system is present in flagella, and that increase of energy supply at mitochondria by decrease of CO_2 may possibly contribute to the initiation of trout sperm motility.

MATERIALS AND METHODS

Mature male rainbow trout (*Salmo gairdneri*) was obtained from Oshino Branch of Yamanashi Prefectural Fisheries Experimental Station. They were kept in an aquarium with circulating and aerating water at 10°C. The semen was collected by inserting a pipette into the sperm duct. Collected sperm was preserved on ice without dilution for several hours during the experiments.

For investigating the effects of K^+, dilution, inhibitors of respiratory chain and uncoupler of oxidative phosphorylation on sperm respiration and motility (Figs. 1–3), 100 mM NaCl or KCl solution was kept without bubbling with any gases. With 3 ml of the above solutions 0.1 ml semen was diluted with various conditions in the chamber of oxymeter and oxygen consumption was measured. Each plot in Figures 2, 3 and 5 was calculated from the oxygen consumption in 5 sec after dilution. On a glass slide without cover 0.1 μl of semen was suspended in 50 μl of 100 mM NaCl solution and sperm motility was observed by light microscopy using dark illumination. NaN_3 and KCN were each dissolved in distilled water. CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) was dissolved in 4% ethanol which did not affect sperm motility and respiration.

For studying the effect of O_2 (Fig. 4a), N_2 gas was introduced into 200 ml of 100 mM NaCl solution in an Erlenmeyer flask from a N_2 gas cylinder. Amount of dissolved O_2 was checked at an appropriate time interval with an oxymeter. A closed chamber (Bellco: 0.75 ml) was filled with the solution containing various concentrations of O_2 using a syringe and 1 μl of semen was injected with a microsyringe, and then the motility of sperm in the chamber was observed under microscope.

The effect of completely-O_2-eliminated condition (Fig. 4b) was investigated in 100 mM NaCl solution containing various concentrations of $Na_2S_2O_4$, which was introduced into both a closed chamber and an oxymeter, and sperm motility and oxygen content were measured.

Effect of CO_2 on sperm motility was investigated (Fig. 5) as follows. CO_2 gas was bubbled into 200 ml of 100 mM NaCl solution in the flask for a few hours. pH value of the solution decreased during CO_2-bubbling and finally reached 6.0. Media containing various concentrations of CO_2 were prepared with mixing the CO_2 saturated medium with 100 mM NaCl solution and pH was adjusted to 6.0 with HCl. Each medium was introduced into the closed chamber and oxymeter, and sperm motility and oxygen consumption were measured. Amount of CO_2 and pH value in these media were checked with a carbon analyzer (Model 524 C, O. I. Corporation, U. S. A.) and pH meter respectively before experiment.

Oxygen consumption was measured with an oxymeter (Yanagimoto Co., Ltd.) for 30 to 60 sec at a chart speed of 30 or 60 cm/min. Solutions were buffered with 20 mM Hepes-NaOH at pH 8.0 (Figs. 1–4) and 6.0 (Fig. 5). Experiments were carried out at 10°C (Figs. 1–4) or 20°C (Fig. 5).

Tracks of sperm were recorded by VTR through a video camera connected with a microscope and percentage of motile sperm and swimming speed in Figures 4 and 5 were measured as described previously [7]. In Figures 1 and 3, the number of moving spermatozoa was evaluated in terms of grade (−, ±, +): grade +, at least over half of spermatozoa were motile in the field of view of microscope; grade ±, below half of spermatozoa were motile; grade −, all spermatozoa were immotile.

RESULTS

Effect of potassium

As shown in Figure 1, when spermatozoa were suspended into 100 mM NaCl solution at a dilution ratio of 1:30, in which spermatozoa initiated forward motility, they consumed oxygen at the rate of

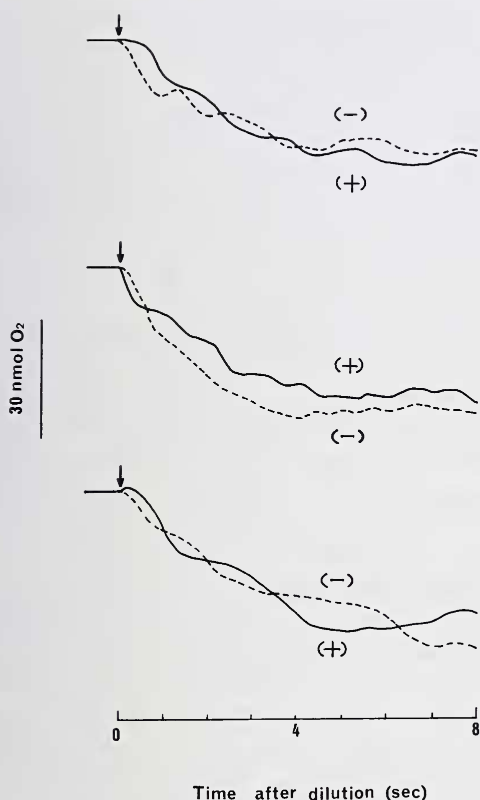

FIG. 1. Change in the oxygen consumption of rainbow trout spermatozoa in NaCl and KCl solutions. Semen at the volume of 0.1 ml was diluted with 100 mM NaCl solution (——) or 100 mM KCl solution (- - -) buffered with 20 mM Hepes at pH 8.0. Arrows indicate the time of adding the semen. Sperm motility was exhibited in parentheses.

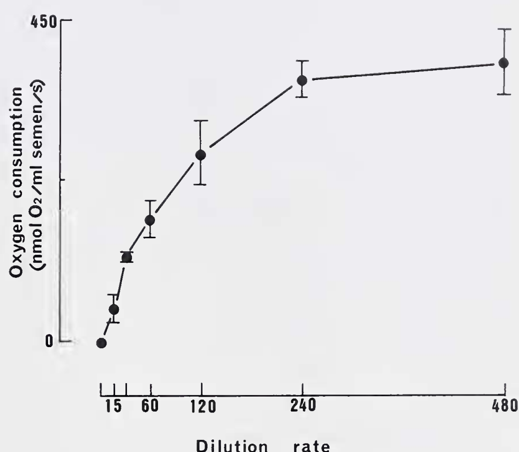

FIG. 2. Effect of dilution on the oxygen consumption of rainbow trout spermatozoa. The appropriate volume of semen was added to 3 ml of 100 mM NaCl solution buffered with 20 mM Hepes, pH 8.0. Vertical bars represent Means±S.E. in 3 experiments.

66.9±3.8 nmol/ml semen/sec from three experiments in Figure 1 in 5 sec after dilution and then the rate decreased. Spermatozoa diluted in 100 mM KCl (1:30 dilution) were completely immotile, however, they consumed oxygen at 67.1± 11.7 nmol/ml semen/sec in 5 sec and then the oxygen consumption became lower. Namely the rate of oxygen uptake of the sperm which were quiescent in the presence of K^+ was almost the same as that of the sperm which initiated motility in the absence of K^+.

Effect of dilution

Oxygen consumption of undiluted trout semen was almost zero (Fig. 2). When semen was diluted in 100mM NaCl solution (1:15 dilution), spermatozoa consumed oxygen at 45±20 nmol/ml semen/sec. Oxygen consumption increased with the increase of a dilution rate and reached almost maximum at a dilution rate of 1:240 (365±26 nmol/ml semen/sec). The level was maintained until a dilution rate reached 1:480.

Effects of NaN_3, KCN and CCCP

Oxygen consumption of spermatozoa in 5 sec in 100 mM NaCl solution at a dilution rate of 1:30 was 69.4±6.2 nmol/ml semen/sec (Fig. 3a), which was almost equal to that in Figure 1. When the dilution medium contained NaN_3, oxygen consumption of sperm decreased with the increase of concentration of NaN_3: In the medium containing 10 mM NaN_3, it was 67% of that in the NaN_3 free medium. Sperm motility was almost suppressed with 5 mM NaN_3 and completely suppressed with 10 mM NaN_3.

Oxygen consumption and motility of the sperm decreased as the concentration of KCN increased (Fig. 3b). The oxygen consumption reached to 67% of that in the KCN free condition in the presence of 10 mM KCN. Sperm motility became feeble by the addition of 5 mM KCN and was completely suppressed by 10 mM KCN.

As shown in Figure 3c, when spermatozoa were diluted with 100 mM NaCl solution containing